

Report of Drs. Cole and Avery (assisted by Drs. Dubos, Goebel, Goodner,  
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1. The chemical and specific properties of pneumococcus antibodies.
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9. Conjugated carbohydrate-protein antigens containing glucuronic and galacturonic acids.

The chemical and specific properties of antibodies and of the factors which condition their behaviour. (Goodner and Horsfall) A. The protective action of antibodies. The work on this subject has been greatly stimulated by the recognition that immune sera from animals of different species, although produced with the same antigen and reacting specifically with this antigen, may differ greatly in their immunological properties. For instance, the antibodies present in antipneumococcus horse serum differ in size, solubility, and degree of specificity from those in anti-pneumococcus rabbit serum. The optimum physical conditions for the interaction be-

tween antigen and antibody differ for the two kinds of sera and the immune precipitates formed with the specific polysaccharides are distinctive in appearance. Evidence has been presented which suggests that the antibody in horse serum is a lecithoprotein, while that in immune rabbit serum is possibly a cephaloprotein. Indeed, it has been shown that these antisera differ in at least thirty respects.

An intensive study has been carried out as to the mouse protective value of nine antipneumococcus sera with reference to their apparent antibody content as determined by in vitro methods. The amounts of "antibody nitrogen" were determined by the method of Heidelberger and Kendall. The mouse protective values were obtained with the method of Kirkbride and Hendry, the results being analysed according to the Maunch 50 per cent end-point system. From the data summarized in Table I it will be noted that the results lend themselves to an arrangement by groups.

Table I

The Protective Values of Antipneumococcus Sera with Reference to Antibody Content

Group	Serum	Nature of Serum	Protective units per cc. of serum	Specifically precipitable nitrogen mg/cc	Protective units per mg. precipitable nitrogen
I	1	Horse - raw	440	0.894	550
	2	" "	555	1.02	550
	3	" "	760	1.50	510
	4	" concentrated	3145	5.75	550
II	1	" raw	1000	1.167	860
	2	" concentrated	6100	7.125	855
III	1	Rabbit - raw	1070	0.938	1140
	2	" "	1830	1.40	1300
	3	" "	1910	1.51	1265

In each of these groups are included sera which have similar protective properties with reference to the amount of antibody nitrogen. Thus Group I comprises those horse sera which show approximately 550 protective units per mg. of specifically precipitable nitrogen. In our experience the majority of horse sera, both raw and concentrated, fall into this group. The two horse sera of the second group show a relatively high protective index and are to be regarded as exceptional and atypical. The immune rabbit sera form a group unto themselves and are characterized by high protective index.

It seems therefore, that the amount of protein precipitated by means of the specific polysaccharide is not an accurate index of the amount of specific antibody. The amount of protein precipitated is dependent to some extent on physical conditions such as time, temperature, dilution, etc. Furthermore, just as it has been shown that lipids are readily adsorbed by these immune precipitates, so too is it possible that various proteins, other than the specific antibody, may also be adsorbed. It has been shown that precipitates frequently contain not only the specific antibody but also other antibodies which happen to occur in the serum.

The higher protective ratios in the case of rabbit sera are partially explicable on the basis that the antibodies in immune horse serum are several times larger than those in immune rabbit serum and consequently the proportion of surface to mass is much smaller.

The immune sera may contain mixtures of antibodies of various capabilities. This possibility is supported by the following points:

a. From antipneumococcus horse serum there has been isolated in a state of immunological purity, i.e., complete precipitability upon the addition of the capsular polysaccharide, a substance of the nature of a lipo-protein which gives a protective value of 1550 units per mg. of

specifically precipitable nitrogen. This material contains 4.13 per cent lipid, has the properties of an euglobulin and reacts rapidly with the polysaccharide in the formation of immune precipitates. It will be noted that this protective value is about twice that of the antibody of the best unpurified horse serum and three times that of the usual immune horse serum.

b. In addition, a second form of antibody has been obtained. Its precise nature is as yet uncertain because in the form now at hand it is only 92 per cent precipitable. However, the properties of this material are very different from those of the first isolated antibody. This second substance is water soluble (except at its isoelectric point), behaves as a pseudo-globulin toward ammonium sulphate, reacts slowly with the polysaccharide, in contrast to the first described antibody, and has a lipid content of approximately 3 per cent. Moreover its protective qualities are low, about 300 units per mg. of precipitable nitrogen.

B. Antipneumococcus rabbit serum as a therapeutic agent in lobar pneumonia. (Horsfall and Goodner) With increased knowledge of the contrasting properties of antipneumococcus horse and rabbit sera, it became apparent that the latter might prove the more useful therapeutic agent in human lobar pneumonia. The indications for its use were as follows:

1. The specific antibodies in antipneumococcus rabbit serum are much smaller than those in antipneumococcus horse serum as judged both by ultrafiltration and by ultracentrifugation. Since the infective processes of lobar pneumonia and many of its complications are characteristically of an extra-vascular order, it is necessary that the antibodies pass through the vascular membranes. Other factors constant, the smaller the antibody the more effective should be its penetration into the tissues.

2. Under certain circumstances antipneumococcus horse serum confers no protection to mice. Thus with massive infections, if amounts of

serum in excess of a definite optimum are used, the antibodies are ineffective. In proportion to body weight these amounts of serum are not greater than have occasionally been administered to patients. This prozone effect is not obtained with antipneumococcus rabbit serum. Secondly, the protective action of immune horse serum is completely abolished by the addition of small amounts of either cholesterol or cephalin. These lipids do not effect the protective action of the rabbit antibody. It seemed possible that this situation might be operative in the specific therapy of lobar pneumonia in certain types of patients.

3. Immune rabbit sera are much more easily produced. For any given type of pneumococcus an effective serum may be produced in rabbits in as short a period as four weeks whereas many months are required with the horse. Furthermore, the cost of producing rabbit serum is much less, (about one-fifth) than that of producing concentrated horse serum. Because raw immune rabbit sera possess high protective potencies, concentration is not so important as in the case of horse serum. Practically all rabbits will produce serum of high potency while many horses will not produce effective serum.

Since there seemed to be no obvious contraindication to the use of type-specific antipneumococcus rabbit serum in human lobar pneumonia, this therapeutic agent was first used in those types with which a considerable experience had been obtained with the corresponding horse immune serum. Twenty-two patients with lobar pneumonia have now been treated. By types these are divided as follows: Type I, 10 patients; Type II, 4 patients; Type VII, 3 patients; and Type VIII, 5 patients. In twelve of the 22 patients, positive blood cultures were obtained before serum was administered, in seven there was consolidation of two or more lobes, and

in three, pleural exudates containing large numbers of pneumococci were present.

Of these twenty-two patients, twenty-one recovered. In the fatal case, death occurred following rupture of the aorta five weeks after the onset of the disease. Crisis occurred on an average 26 hours after the institution of serum therapy. With improvements in the technique of serum administration this time interval has become much shorter, the average for the last five cases being 9 hours.

The average total amount of immune rabbit serum administered, irrespective of the type, was 159 cc. per case. It is of interest that the average dosage in the ten Type I patients was 124 cc. whereas in the last 437 patients treated with unconcentrated Type I horse serum, the average dosage was 440 cc.

In the first cases treated, chill reaction not infrequently followed the administration of rabbit serum. It was found, however, that the frequency and severity of these reactions were much less if the serum employed had been heated to 56° and adsorbed with sterile kaolin. Finally it has been found that the chills may be entirely prevented if acetylsalicylic acid be administered simultaneously with the first injection of this heated and adsorbed serum. On the other hand acetylsalicylic acid is not able to prevent chills following the use of untreated serum. In no cases have anaphylactoid reactions occurred.

By combination of the procedures mentioned, it has been possible to entirely eliminate any untoward symptoms in the recent cases.

Recovery without operation occurred in two patients with grossly infected pleural exudates, and it was possible to demonstrate that the fluid from these cases contained type specific antibody of rabbit origin. In one

patient, antibody was not demonstrable in the pleural fluid, and empyema subsequently developed.

This brief summary of the results obtained in a short series of severe cases does not adequately convey the favorable clinical impression which the use of anti-pneumococcus rabbit serum has created. It is hoped that additional experience will confirm the impression now held that the employment of rabbit serum marks a distinct advance over the use of horse serum in the treatment of lobar pneumonia.

The autolytic system of pneumococci. (Dubos) (a) Relation to the antigenicity of the cells. Under appropriate conditions, the immunization of rabbits with suspensions of virulent, encapsulated pneumococci brings about the production of type specific antibodies directed against the capsular polysaccharides of the organisms used as antigens. However, it is known that when pneumococci are disintegrated by autolysis, by treatment with bile salts, or by freezing and thawing, they fail to incite rabbits to produce these particular antibodies. It has been found that, to be soluble in bile, or to be liable to disintegration by repeated freezing and thawing, pneumococci, living or dead, must still possess in an active form some, at least, of their autolytic enzymes; furthermore, the disintegration can take place only when conditions are favorable for enzymatic action. It appears therefore that all methods commonly used to achieve a dissolution of pneumococci involve as a necessary step the action of at least one or a few of the enzymes which constitute the autolytic system of the cells.

A study has been made of the action of purified preparations of the autolytic enzymes upon pneumococci and other substrates. It has been found in particular that these enzyme preparations are capable of inactivating the capsular polysaccharide antigens of virulent pneumococci. To achieve this inactivation, it is not necessary to bring about a complete dissolution of

the bacterial cells; it is merely sufficient to cause a limited alteration of the cellular structure such that the latter has lost its ability to retain the Gram stain, but still retains its essential morphological characteristics. The structure which carries the capsular polysaccharide antigen which determines the Gram positive reaction of the cell has been found to be destroyed by the bacteriolytic enzyme but to be resistant to common proteolytic and lipolytic enzymes. It has a low nitrogen content (about 8%) and accounts for less than 25 per cent of the original weight of the cell.

The bacteriolytic enzyme also possesses the property of releasing reducing sugars from acetyl amino glucose glucuronides of animal and bacterial origin which are otherwise very resistant to enzymatic action.

It is hoped that a continued study of the mechanism of action of the bacteriolytic enzyme may reveal information related to the antigenic structure of pneumococci. But in any case, the fact that this enzyme is capable of destroying the type specific antigenicity of this group of microorganisms does emphasize the importance of minimizing the chances of alterations due to enzymatic action in the course of preparation of bacterial antigens.

(b) The action of tissue enzymes upon bacteria (Dubos) The immunization of rabbits with heat killed pneumococci by the intradermal route fails to incite the production of the type specific carbohydrate antibodies which would follow immunization by the intravenous route. This lack of antigenic response suggested the existence in the skin of a mechanism capable of inactivating the capsular polysaccharide antigen of pneumococci.

In fact it has been possible to isolate from different tissues and from leucocytes an agent endowed with the following activities; a) it decomposes yeast nucleic acid without, however, releasing free phosphoric



acid in solution, b) it attacks heat killed bacteria (pneumococci, streptococci, staphylococci) and yeasts, destroying their affinity for the basic dyes but without causing a disintegration of the cellular structure, c) it renders ineffective in rabbits the type specific antigen of pneumococci.

The agent responsible for these different types of action appears to be a protein with enzyme-like properties which is rapidly inactivated by pepsin, and more slowly by trypsin. It is very resistant to heat, especially at acid reactions. The resistance to heating is explained by the fact that the agent is denatured at temperatures above 80°C, but that the denaturation is rapidly reversed on cooling.

It appears therefore that there exist in tissues, agents of an enzyme-like nature which are heat resistant, and capable of attacking and modifying the antigenicity of injected materials.

(c) The production of specific enzymes by bacteria as a tool in biochemical analysis. (Dubos) In previous years a technique has been described for the production of a bacterial enzyme capable of decomposing the capsular polysaccharide of Type III pneumococcus. This enzyme exhibits a remarkable specificity since it does not decompose any other polysaccharide tested, not even the capsular polysaccharide of Type VIII pneumococcus which is very closely related to Type III polysaccharide both chemically and serologically. The production of the enzyme also exhibits an interesting character of specificity. Whereas the microorganism which produces it can grow readily and abundantly on all sorts of laboratory media, it fails to produce the specific enzyme except when compelled to utilize the Type III polysaccharide in the course of its growth.

These observations suggested the possibility of developing a method for the production of enzymes specifically directed against definite

There are no satisfactory chemical methods for the determination of creatinine in tissue and body fluids. It was therefore of interest to obtain an enzyme capable of decomposing this substance. To this end, four bacterial species capable of attacking creatinine were obtained in pure culture. Cultures of one of these species has yielded an enzyme preparation capable of decomposing creatinine into urea and probably sarcosine. The action of this enzyme is specific. Of all the other substrates tested, only creatine, glycoylamidine and 5-methyl glycoylamidine, substances closely related to creatinine, are also attacked.

The bacterial culture grows readily on peptone solutions but interestingly enough it produces the specific enzyme only when compelled to utilize creatinine in synthetic media. The situation is therefore comparable to that described for the production of the enzyme which decomposes the capsular polysaccharide of Type III pneumococcus.

It appears therefore that a method is available for the production of reagents specific for substances of biological interest. In collaboration with Dr. Miller, the new enzyme preparation is being employed for physiological studies on the presence of creatinine in tissues and body fluids, and for a measure of renal function.

The transformation of specific types of pneumococcus. (MacLeod)

In the reaction system used in the transformation of pneumococcal types in vitro, three components are required in addition to the broth used as culture medium; 1) living R pneumococci, 2) the specific transforming extract prepared from smooth pneumococcal cells - the bacterial factor, and 3) serum known to contain an accessory factor, serum factor.

Further observations have been made on some of the properties of the specific or bacterial factor. It has been found to be extremely labile

if exposed to ultra-violet light, but resists oxidation as by the action of iodine or exposure to atmospheric air in a shallow layer at 37°. The specific agent is more stable to heat at acid reaction in a solution containing 50 per cent ethyl alcohol than if heated in aqueous solution under the same conditions. Enzyme preparations known to be high in phosphatase activity, such as bone phosphatase preparations, very quickly inactivate the specific bacterial factor, but it is not held from the evidence that the specific agent is necessarily of the nature of a phosphoric ester, inasmuch as such enzyme preparations probably contain other enzymes than the predominant phosphatase. Further precipitation of bacterial extracts has been accomplished by deproteinization by the use of chloroform and amyl alcohol after the method of Sevag, followed by repeated fractionation in alcohol. However, no selective means of isolating the specific fraction has yet been discovered.

The third component of the reaction system used in the transformation of pneumococcal types, namely the accessory factor present in serum, has been more extensively studied. It has been shown previously that rabbit serum, normal swine serum, and human ascitic or pleural fluid contain the necessary serum factor. However no serum or fluid has yet been found to function in the transforming system that has not contained agglutinins for R pneumococci. This fact suggests that the R antibodies are in some way related to, if not responsible for the accessory action of serum in the transforming process. When serum is fractionated the accessory factor and also the antibodies are contained in the globulin fraction. That complement is not concerned in the reaction is shown by the fact that heating at 60°C for 30 minutes, which completely inactivates the complement, does not impair the capacity of serum to function as accessory substance. Under these conditions the R antibodies remain unaffected. These substances and also the

accessory factor are destroyed when serum is heated at  $70^{\circ}\text{C}$ , a temperature at which the serum proteins are denatured. Although the presence of R antibodies and the accessory factor of serum are intimately associated, it does not necessarily follow that they are identical. While it is true that no serum has functioned as accessory substance which has not contained demonstrable R antibodies, the converse of this does not hold true, namely, that all sera containing these antibodies necessarily act in the transforming system. It has been of common occurrence, when different sera of equal anti-R titers are tested, to find that one may function and another completely fail to act as accessory factor in the reaction of transformation.

The evidence so far obtained therefore, indicates that the serum factor necessary in the transformation reaction is contained in the globulin fraction, is not identical with complement, and that it is relatively stable, resisting heating at  $60^{\circ}$  for 30 minutes.

All sera, moreover, are not effective in this reaction. It has been found that while sera of patients actively ill with pneumonia or other acute infections, may function as the serum factor in the transformation, sera from normal persons and persons during convalescence from pneumonia fail to act. It has further been found that normal and convalescent sera not only fail to act, but when these sera are added to the bacterial transforming agent, they so modify the latter that no transformation occurs even when serum known to contain the accessory factor is added.

When normal or convalescent serum is heated to  $60^{\circ}\text{C}$  at an alkaline reaction, which temperature does not destroy the serum factor, the heated serum is as effective in promoting the transformation reaction as is serum from acutely ill patients. It is evident, therefore, that there is something in normal serum which destroys or prevents the action of the bacterial factor and that this agent in normal serum may be destroyed by heating at  $60^{\circ}$

at an alkaline reaction. It seems not improbable that this substance is of the nature of an enzyme.

This enzymatic substance, however, may be isolated from all human serum, whether normal or serum obtained during the acute phase of pneumonia. Isolation is accomplished by alcohol fractionation of the serum. The isolated enzymatic fraction of acute phase serum inactivates the transforming principle although the whole serum from which the fraction was derived does not do so. All human sera therefore, not only contain the accessory factor necessary for transformation, but they are all potentially able to destroy the specific bacterial factor.

The reason that acute phase serum is active in the transforming process while convalescent or normal serum is not, is therefore probably to be explained by the presence in acute phase serum of an antiferment which is absent from normal serum.

Studies on the antigenic mosaic of pneumococcus. (Avery, Geobel, and MacLeod). In the course of experiments on the nature of the capsular antigen of Type I pneumococcus, a hitherto unrecognized fraction of the cell has been isolated. This material, designated the "Z substance" is tentatively regarded as a protein which in many of its chemical properties resembles the alcohol soluble globulins, the so-called protamines, of plant tissue.

The Z substance in the present state of purity contains approximately 15 per cent nitrogen, 2 per cent sulfur, but no phosphorus. In aqueous solution this substance gives the usual protein color reactions, and is precipitated from solution by trichloroacetic acid, picric, phosphotungstic and sulfosalicylic acids. In the form of the hydrochloride it is soluble in 80-90 per cent alcohol and as the picrate it is soluble in 70 per cent acetone. The solubility of Z in acidulated alcohol and its property of forming acetone-soluble picrates have been utilized in devising methods for

extracting the material from the bacterial cells. The substance has a sharp isoelectric point, precipitating completely from solutions adjusted to pH 4.7.

The Z substance derived from Type I pneumococci is antigenic in rabbits and gives rise to the formation of type specific antibodies. The serum of immunized rabbits agglutinates Type I cells and confers specific protection on mice against infection with virulent organisms of the homologous type. Interestingly enough, however, this immune serum does not react in precipitin and complement fixation reactions with Type I capsular polysaccharide.

Further work is in progress on the chemistry and immunology of this particular fraction which appears to be concerned in the type specific mechanism of the cell.

Comparative virulence of pneumococci implanted on the respiratory mucous membranes of mice by the inhalation method. (Stillman). In order to determine what importance the virulence of the infecting organism plays in the severity of the actual disease, the relative invasiveness of different strains of freshly isolated pneumococci has been tested in mice by the inhalation method. As soon as possible after isolation of a culture from a patient with lobar pneumonia, the virulence of the strain has been tested by spraying 10 mice with the organisms. At the same time virulence has been determined by injecting mice intraperitoneally with amounts representing  $10^{-5}$  and  $10^{-6}$  cc. of the original culture. In the case of the mice exposed in a closed chamber to an atmosphere laden with Types I or II pneumococci, no infection resulted unless the animals were intoxicated with alcohol just prior to exposure. It has been found that freshly isolated Type I pneumococci have little invasive virulence for mice as tested by implantation on the mucous membranes by inhalation. Indeed, even when injected intraperit-

oneally in the dilutions used only an occasional strain proved fatal.

Type II organisms, on the contrary, were quite invasive when tested by inhalation and regularly killed mice when injected intraperitoneally. The Type III and VIII strains showed great variations. Although they were uniformly virulent when tested intraperitoneally, only a few strains were found to invade by way of the mucous membranes, the majority of cultures failing entirely to incite infection when introduced by this route.

The clinical course of the spontaneous disease in patients is being correlated with the virulence of the infecting pneumococci as tested in mice by the inhalation method.

Specificity of conjugated carbohydrate-protein antigens containing glucuronic and galacturonic acids. (Goebel and Hotchkiss). The uronic acid constituent of the encapsulating polysaccharides of virulent pneumococci appears to be of special significance in determining serological properties. In certain of these type specific substances, glucuronic acid is found as an integral part of the molecule while in others galacturonic acid forms a part of the encapsulating material. It was of interest therefore to compare the immunological properties of artificially compounded antigens containing glucuronic and galacturonic acids, since the stereochemical configuration of these two substances is known. In this manner it should be possible to establish directly the relationship between the chemical constitution of these biologically important acids and the specificity of the antibodies to which they give rise.

When rabbits are immunized with azo-protein antigens containing glucuronic and galacturonic acids, the antibodies elicited are specific and show no serological crossing. Since the configuration of the uronic acid radical of each antigen differs only by an interchange of the H and OH groups on the fourth carbon atom, the sharply defined differences in immunological properties must be directly attributable to this fundamental difference in chemical configuration.

From a comparison of the immunological properties of antigens containing the benzyl glycosides of glucose and galactose with antigens containing the hexose uronic acids, it has now been found that each gives rise to antibodies which are specific and which show no serological crossing. Differences in the immunological properties of these sugar and sugar acid antigens is consequently attributed to differences in polarity of the grouping occupying the sixth position in each carbohydrate radical as well as differences in configuration within the sugar molecule. In the glucoside and galactoside molecules this grouping is a primary alcohol ( $\text{CH}_2\text{OH}$ ), whereas the polar carboxyl group ( $\text{COOH}$ ) occupies this position in the glucuronide and galacturonide. Thus a new and important factor must be taken into consideration in understanding the specificity of simple carbohydrates and their uronic acid derivatives, namely the polarity of groups in the molecule.

The artificial azo-protein antigens containing glucuronic and galacturonic acids bear a striking immunological relationship to the specific pneumococcus polysaccharides, for the antigens, in dilutions as high as one part in a million, precipitate in antipneumococcus sera. The hexose antigens, containing glucose and galactose, on the other hand show no serological activity in these same antisera.

It has been found by Dr. Hotchkiss that synthetic high molecular polymers bearing carboxyl and hydroxyl groups as substituents upon a long chain of carbon atoms precipitate specifically in antipneumococcus horse serum Type III. The reaction still occurs at a dilution 1:300,000 of the polymer, but there is no precipitation even in much higher concentrations in antipneumococcus serum Type III previously absorbed with homologous polysaccharide or in unabsorbed Types I and II immune sera. The reactive synthetic substances were prepared by the polymerization of acrylic acid with



vinyl acetate, followed by saponification of the acetyl groupings. These haptens, in contrast to the azo-proteins, are entirely synthetic and their chemical constitution is quite well understood.

The precipitation of uronic acid azo-protein antigens and of synthetic acidic polymers in antipneumococcus sera is believed to be caused by a neutralization of the charge of the basic groups of the antibody molecule by the acidic group of the antigen. This hypothesis is in part substantiated by the fact that when the amino groups of pneumococcus antibody are acetylated, the resulting acetylated protein fails to precipitate any of the acidic antigens. This mechanism is in part believed to be the reaction underlying the combination of antibody with homologous type specific pneumococcus polysaccharides. The specificity of this phenomenon may possibly be governed by the arrangement in space of the polar groups of the hapten molecule, which in turn condition the spatial pattern of the reactive groups of the antibody molecule.

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